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Elevated pro-inflammatory cytokines and chemokines in saliva of cats with feline odontoclastic resorptive lesion

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ABSTRACT

Feline odontoclastic resorptive lesion (FORL) is an inflammatory oral disease of unknown aetiopathogenesis that affects between 20% to 75% of cats. Twenty immune-associated molecules were measured in saliva of 25 healthy and 40 cats with FORL using a multiplex assay. No statistically significant differences were observed in the levels of these proteins between the healthy group and the diseased group of cats. A two-step cluster analysis of the oral microbiome and salivary cytokine data identified two subgroups of cats with FORL: FORL-1 (subset of cats with a less diverse oral microbiome) and FORL-2 (diseased cats with a microbiome similar to that of healthy animals). The level of some key proinflammatory cytokines (IL-1β, IL-12p40) and chemokines (IL-8, RANTES, KC) were significantly higher in the FORL-1 subgroup than in the FORL-2 subgroup and the healthy group. In addition, TNF-α levels were greater in the FORL-1 subgroup than in the FORL-2 subgroup. These increases in pro-inflammatory cytokines and chemokines indicate active ongoing inflammation that may promote the osteoclastic/odontoclastic activity associated with FORL.

Feline odontoclastic resorptive lesion (FORL) is a chronic inflammatory oral disease accompanied with severe pain leading to tooth resorption, affecting over 50% of cats over the age of one year. Its aetiology is unknown, although inflammation is thought to be one of the initiating causes of FORL that leads to destruction of affected teeth (DeLaurier et al., 2002; Reiter et al., 2005; Booij-Vrieling, 2010; Thomas et al., 2022).

Salivary levels of inflammatory mediators are considered as biomarkers for periodontal diseases (Jaedicke et al., 2016; Belstrøm et al., 2017). Periodontal disease is accompanied by inflammation (Healey et al., 2007; Kirkwood et al., 2007) which is associated with alveolar bone resorption in humans (Kesavalu et al., 2006; Meka et al., 2010), horses (Kennedy et al., 2017) and tooth resorption in dogs (Riggio et al., 2011; Marshall et al., 2014; Wallis et al., 2015; Nises et al., 2018) and cats (Girard et al., 2009; Mata, 2015; Perry and Tutt, 2015). Inflammatory mediators initiate osteoclastic/odontoclastic activity in these diseases (Fuss et al., 2003; Booij-Vrieling, 2010; Hienz et al., 2015).

While many bacteria reside in the oral cavity as commensals, the oral mucosa is repeatedly exposed to minor injury as part of chewing process. This provides an entry point for invasive pathogenic bacteria which can initiate inflammatory and immune responses by TLR activation, leading to release of chemokines and cytokines which, in turn, recruit and stimulate leukocytes to promote osteoclast activity via the RANKL pathway (Boyle et al., 2003; Park et al., 2017). Pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α promote osteoclast/odontoclast activity whilst the anti-inflammatory cytokines IFN-γ (Th1) and IL-10 cytokine suppress their activity (Amarasekara et al., 2018).

Cytokines present in the local tooth microenvironment may play an important role in the pathogenesis of FORL. Therefore, the aim of this study was to evaluate the role of salivary cytokines/chemokines as markers of inflammation in the pathology of FORL.

Saliva samples from 25 healthy cats and 40 cats with FORL were collected using commercially available Salivette® (Sarstedt, Nümbrecht, Germany) following the manufacturer's instructions. Cats were allowed to chew on the Salivette for 10–15 s. The Salivettes were centrifuged (1000 ×*g* for two minutes). Approximately 100 μL of saliva was collected from each cat and stored at -80 °C.

Cytokines were assayed using the Milliplex MAP Feline Cytokine/Chemokine Magnetic Bead Panel Premixed 19 Plex kit (Millipore Sigma, Burlington, MA USA). Saliva samples were assayed neat in duplicates.

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Table 1
Number of teeth affected in cats in FORL-1 and FORL-2 subgroups.

FORL-1 subgroup		FORL-2 subgroup	
Cat ID	Number of teeth affected	Cat ID	Number of teeth affected
BB0027FM	3	BC0007FF	1
BE0058FM	1	BE0087FM	2
BC0015FM	5	BE0075FF	1
BB0001FM	2	BC0066FM	1
BB0022FF	4	BB0006FM	1
		BB0061FM	1
		BC0008FM	2
		BC0031FM	1
		BB0048FM	2
		BD0127FF	3
		BD0118FM	1
		BD0003FM	1
Average number of teeth affected	3 ± 1.6	Average number of teeth affected	1.5 ± 0.7

The biomarker panel included sFas, Flt-3 ligand, GM-CSF, IFNγ, IL-1β, IL2, IL-4, IL-6, IL-8, IL-12(p40), IL-13, IL-18, KC, MCP-1, PDGF-BB, RANTES, SCF, SDF-1 and TNF-α.

Cytokine data from healthy and cats with FORL was compared using independent samples *t*-test (GraphPad Prism 6.0). Differences with significance levels of $p \leq 0.05$ were considered significant. A two-step cluster analysis of the salivary immune markers along with the oral plaque microbiome (Thomas, 2019; Thomas et al., 2021) was carried out using SPSS Version 21. The clusters were then analyzed by ANOVA (SPSS Version 21).

The study included 65 domestic short hair, spayed or neutered cats. The mean age of cats with FORL was 7.2 years (male $n = 28$, mean age 8.05 years; female $n = 12$, mean age 6.83 years). The mean age of healthy cats was 4.9 years (male $n = 14$, mean age 4.28 years). These were the same set of cats used in a previous microbiological study (Thomas et al., 2021).

Radiography confirming stages of FORL was available for 18 of the 40 cats with FORL (5 in the FORL-1 subgroup and 13 in the FORL-2 subgroup). One cat in the FORL-1 subgroup had the highest number of teeth extracted (five) due to FORL (Table 1).

Salivary inflammatory biomarkers in cats with FORL differentiated into two groups. The first group included IL-1β, TNF-α, IL-8, RANTES (CCL5), KC (CXCL1), IL-2, IL-12p40, IL-18, IFN-γ, IL-4, Flt-3 ligand and FAS (data not shown). Salivary cytokines in the first group appeared to be higher in several cats with FORL, however the differences were not statistically significant. The second group of cytokines included IL-6, IL-13, GM-CSF, PDGF-BB, SCF, MCP-1 and SDF-1 (data not shown) and did not show increased levels in the saliva from cats with FORL in comparison with salivary cytokines from healthy cats. A two-step cluster analysis was carried out using a combination of salivary inflammatory biomarkers and oral microbiome data (Thomas et al., 2021) from the same set of cats. Following this analysis, the cats with FORL separated into two subgroups, namely FORL-1 and FORL-2 (Fig. 1). The FORL-1 subgroup of cats had a lower microbiome diversity than both the FORL-2 subgroup and healthy group (Thomas et al., 2021), perhaps due to differences in severity of disease. The FORL-2 subgroup clustered closer to the healthy cats, and they had microbiomes of similar diversity. Salivary cytokines were compared between the FORL-1 and FORL-2 subgroups and the healthy group. Significant differences for the cytokines IL-1β ($p \leq 0.001$), IL-8 ($p \leq 0.001$), IL-12p40 ($p \leq 0.001$), RANTES ($p \leq 0.01$) and KC ($p \leq 0.01$) was noted between the FORL-1 subgroup and the healthy group, and between the FORL-1 and FORL-2 subgroups (Fig. 1). TNF-α showed significant differences ($p \leq 0.05$) between the FORL-1 and FORL-2 subgroups only but did not differ from the healthy group (Fig. 1). No significant differences between any of the groups was observed for the cytokines IL-2, IL-4, IL-6, IL-13, IL-18, IFN γ, Fas, FLt-3 ligand, GM-CSF, PDGF-BB, SCF, MCP-1 and SDF-1 (data not shown). A rank correlation analysis was performed between microbiome species richness and the molecular biomarkers. The following chemokines/cytokines had statistically significant negative correlations (all $p < 0.05$) with species richness: IL-1β (−0.0428), IL-6 (−0.271), IL-12p40 (−0.310), IL-8 (−0.292), KC (−0.326) and RANTES (−0.373).

Salivary cytokines are regarded as biomarkers for periodontal disease (Jaedicke et al., 2016; Belstrøm et al., 2017) and have been successfully detected using Luminex analysis in saliva of patients with oral and systemic diseases (Rathnayake et al., 2013; Rinderknecht et al., 2022). The Luminex kit used in the current study had previously been successfully validated in-house for use with saliva by spiking saliva samples with known amounts of target (data not shown). Cytokines play an important role in the pathological process underlying oral diseases. Increased levels of salivary IL-1β and IL-6 has been associated with the severity of periodontitis in humans (Costa et al., 2010; Gursoy et al., 2011). TNF-α is associated with chronic periodontitis (Sexton et al., 2011). Salivary IL-6 initiates osteoclast differentiation and bone resorption, leading to tissue destruction in peri-implant disease (Liskmann et al., 2006). Several growth factors in saliva, such as platelet-activating factor, vascular endothelial growth factor and hepatocyte growth factor have been correlated with the severity of periodontal inflammation and are important salivary biomarkers of periodontal disease (Rasch et al., 1995; Ohshima et al., 2002; Jaedicke et al., 2016).

Although studies regard salivary cytokines as biomarkers in human periodontal disease, salivary cytokines in FORL have not been studied previously. In this study, salivary cytokines were measured in cats with FORL and healthy cats as markers of inflammation and the initial analysis showed no significant difference in the levels of these key salivary biomarkers. However, the oral microbiome was also evaluated in the same cohort (Thomas, 2019; Thomas et al., 2021). This allowed splitting of the FORL group following a two-step cluster analysis of the oral microbiome and salivary cytokines (Thomas, 2019; Thomas et al., 2021) into FORL-1 and FORL-2 subgroups, with the FORL-2 subgroup clustering closer to the healthy group. Significantly higher levels of the cytokines IL-1β, IL-8, IL-12p40, RANTES (CCL5) and KC (CXCL1) were noted in the FORL-1 subgroup in comparison to the healthy group and the FORL-2 subgroup. TNF-α did not differ between the healthy and diseased group, but it was significantly different between the FORL-1 and FORL-2 subgroups.

Radiographic data evaluating the stages of FORL was available for only 18 cats (five in the FORL-1 subgroup and 13 in the FORL-2 subgroup). One of the cats in the FORL-1 subgroup had five teeth extracted due to FORL. Since an average of 3 ± 1.6 were teeth affected in the FORL-1 subgroup and 1.5 ± 0.7 were affected teeth in the FORL-2 subgroup, this may be an indicator of greater severity of the disease in the FORL-1 subgroup compared to the FORL-2 subgroup. Further studies are needed to confirm to investigate a potential correlation between disease severity and number of teeth affected.

In this study, salivary cytokines from cats with FORL had higher levels of the pro-inflammatory cytokines IL-1β, IL-8, IL-12p40 and TNF-α, and of the chemokines RANTES (CCL5) and KC (CXCL5), thus indicating an ongoing chronic inflammatory response in the oral cavity. Pro-inflammatory cytokines such as IL-1-β and TNF-α (Shiratori et al., 1994) are elevated in saliva of cats with FORL. IL-1β and TNF-α can both induce keratinocyte chemoattractant (KC).

In conclusion, both pro- and anti-inflammatory cytokines were significantly upregulated in a subset of cats with FORL, indicating an active inflammatory response. These findings underscore the relevance of salivary cytokines as biomarkers for inflammation leading to FORL.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

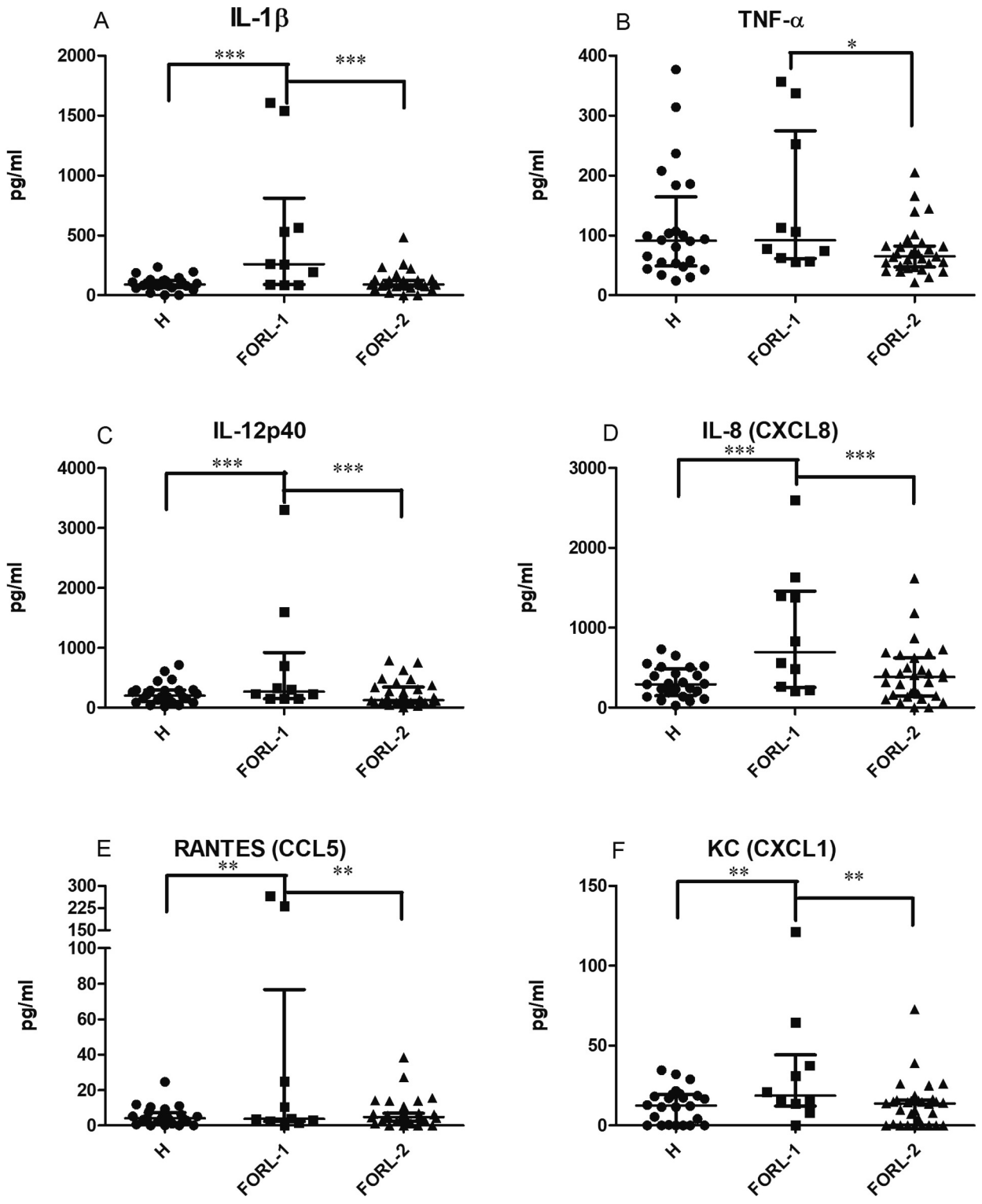

Fig. 1. Salivary cytokine and chemokine levels following two-step cluster analysis.
Cytokines and chemokines levels are indicated in (A) IL-1β, (B) TNF-α, (C) IL-12p40, (D) IL-8, (E) RANTES (CCL5) and (F) KC (CXCL1). X-axis indicates different groups of cats included in the study: FORL-1 ($n = 10$), FORL-2 ($n = 30$) and healthy ($n = 25$). Y-axis indicates concentration of salivary biomarkers (cytokines, chemokines) levels in pg/mL. * $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$.

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